Equine cutaneous mast cell tumours exhibit variable differentiation, proliferation activity and KIT expression

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Abstract: Equine cutaneous mast cell tumours (CMCTs) are generally considered to be benign skin lesions, although recurrent and multicentric tumours have been described. For canine CMCTs, grading and prognostic approaches are well established and aberrant KIT expression as well as high proliferation indices are associated with poor outcome. However, in the case of equine CMCTs, morphological features, proliferative activity and KIT expression pattern have not been assessed or related to biological behaviour, and there is discussion as to whether CMCTs are true neoplastic processes. The present study describes 45 equine CMCTs in terms of their morphology and KIT and PCNA expression by immunohistochemistry. KIT expression was classified as membranous (I), cytoplasmic and focally stippled (II) or diffuse cytoplasmic (III). A large proportion of the tumours were multinodular or diffuse dermal infiltrates of mast cells with mild anisokaryosis, a low proliferative rate and a dominance of KIT pattern I, representing well-differentiated CMCTs. In approximately one third of the cases, the mast cells exhibited more infiltrative growth, moderate to marked anisokaryosis and a higher degree of proliferation. These were classified as poorly differentiated CMCTs and exhibited only KIT patterns II and III. These findings indicate that there is a subgroup of poorly differentiated equine CMCTs, in which there is an association between aberrant KIT expression, high proliferative rate and potential aggressive behaviour, all features that confirm at least the poorly differentiated CMCT as a true neoplastic processes.

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Original Article

Equine cutaneous mast cell tumours exhibit variable differentiation, proliferation activity and KIT expression

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Summary

In horses, cutaneous mast cell tumours (CMCT) are generally considered as benign skin lesions, although recurrent and multicentric tumours have been described. In canine CMCT, grading and prognostic approaches are well established and aberrant KIT expression as well as high proliferation indices are associated with poor outcome. For equine CMCT, however, morphological features, proliferative activity and KIT expression pattern have so far not been assessed and considered in relation to their clinical behaviour, and there is ongoing discussion as to whether CMCT are true neoplastic processes. The aim of the present study was to morphologically characterise equine CMCT and assess their KIT expression and proliferative activity, as tools to identify features that would confirm their neoplastic origin and suggest a variable clinical behaviour. For this purpose, 45 equine CMCT were re-examined to characterise their morphology as well as KIT and PCNA expression, using light microscopy including immunohistochemistry. The KIT expression pattern was classified as membranous (I), cytoplasmic and focally stippled (II), or diffuse cytoplasmic (III). A large proportion of tumours presented as multinodular or diffuse dermal infiltrates of mast cells with mild anisokaryosis, a low proliferative rate, and a dominance of KIT pattern I, representing well differentiated (WD-)CMCT. In approximately one third of the cases, however, the mast cells exhibited a more infiltrative growth pattern, moderate to marked anisokaryosis and a higher degree of proliferation. These were classified as poorly differentiated (PD-)CMCT and also exhibited KIT pattern II and III exclusively. The results of the present study indicate that a subgroup of poorly differentiated CMCT exist, with evidence of an association between aberrant KIT expression, high proliferative rate and a potential aggressive behaviour, all features that confirm at least the PD-CMCT as true neoplastic processes.

Keywords: Equine; mast cell tumours; KIT; mitotic index; PCNA

Introduction

Cutaneous mast cell tumours (CMCT) are relatively uncommon in horses (3.4% of cutaneous neoplasms in a recent survey (Valentine *et al.,* 2006)) however, due to the limited number of publications on equine CMCT, their prevalence might be underestimated. CMCT generally occur in young to adult horses, and predominantly as solitary dermal lesions (Scott & Miller, 2011). They are grossly similar to their canine counterparts and occur as nodular masses with a diameter of up to 20 cm, most frequently on the head, but often also on the trunk and limbs. The overlying skin is intact, alopecic or ulcerated (Kay *et al.,* 2003; Scott &. Miller, 2011).

Histologically, equine CMCT are composed of large single or multiple aggregates of mast cells, intermingled with variable numbers of eosinophils and, occasionally, multifocal areas of collagenolysis (Scott & Miller, 2011). All these features are also observed in other equine skin lesions, among which "equine eosinophilic granuloma" (EEG), a specific chronic inflammatory condition characterised by focal infiltrations of macrophages intermingled with abundant eosinophils and occasionally mast cells (Scott & Miller., 2011), is the most prevalent. However, CMCT are, as the name implies, the only condition that is considered as a neoplastic process, although there are ongoing discussions whether some might represent a reactive process.

Equine CMCT are generally considered as clinically benign, although recurrent and multicentric tumours have occasionally been described (Riley *et al.,* 1991; Tan *et al.,* 2007). In contrast, a recurrence and metastasis rate of approximately 10% and 23% respectively has been reported for the canine counterpart (Kiupel *et al.,* 2011). Attempts to relate prognosis and morphological features grouped canine CMCT in 3 (grades I to III) or, later, 2 ("high" or "low" grade) categories said to predict the clinical outcome in the affected dog (Kiupel *et al.,* 2011; Patnaik *et al.,* 1984).

Development, survival and proliferation of mast cells is regulated by the stem cell factor (SCF), a haemopoietic cytokine that acts as a membrane ligand and interacts with the *c-kit* gene product KIT, a tyrosine kinase receptor also known as CD117 (Wedemeyer *et al.,* 2000). *C-kit* is a proto-oncogene that is expressed in mast cells and several other cells, such as basophils, haematopoietic stem cells, melanocytes and Cajal's cells in the *lamina muscularis* of the digestive tract (Hudson *et al.,* 2001). In canine CMCT, a tandem duplication mutation in the juxtamembrane *c-kit* coding region has been shown to cause activation of the KIT receptor in the absence of SCF (Webster *et al.,* 2004). Studies suggest that this gene may have a substantial role in the neoplastic transformation of mast cells in dogs and have highlighted its usefulness as a prognostic and predictive marker (Webster *et al.,* 2004; Yamada *et al.,* 2011). Immunohistochemical studies have shown KIT expression in both normal and neoplastic canine mast cells, but with different staining patterns and intensity depending on the MCT grades (Reguera *et al.,* 2000); in the Patnaik grading system, grade I tumours exhibit the lowest and grade III the highest staining intensity (Reguera *et al.,* 2000). Also, in well differentiated neoplastic mast cells, KIT expression was found on the cell membrane, whereas the neoplastic cells in high grade tumours exhibited a predominantly intracytoplasmic reaction (Reguera *et al.,* 2000). Indeed, translocation of KIT from the plasma membrane to the cytoplasm was shown to be associated with tumour recurrence and shorter overall survival in the dog (Webster *et al.,* 2004).

So far, studies on the expression of KIT in equine CMCT are very limited. Abnormal cytoplasmic KIT expression in neoplastic cells has been reported in a MCT on the tongue of a mare, without evidence of tumour recurrence or metastatic disease within 8 months after surgery (Seeliger *et al.,* 2007). Also, a recent study did not find any correlation between KIT expression and morphological parameters of malignancy, clinical outcome, or aggressive local behaviour; this was interpreted as evidence of the generally benign behaviour and high degree of differentiation of equine CMCT (Clarke *et al.,* 2014). However, in contrast to this recent publication, in our diagnostic case material, we observed a proportion of equine CMCT with morphological criteria of malignancy. Therefore, we retrospectively examined a cohort of randomly chosen equine CMCT for morphological parameters of malignancy, proliferation indices, and KIT expression.

Materials and methods

Tissues and histological examination

A total of 45 surgically excised and routinely diagnosed equine CMCT (all with >50% mast cells in the infiltrate) were retrieved from the diagnostic database of the Division of Veterinary Pathology, School of Veterinary Science, University of Liverpool (2004-2012) and the diagnosis confirmed by systematic re-examination. For the assessment of morphological criteria, consecutive sections (4 µm) were prepared from each tumour and stained with haematoxylin and eosin (HE) and toluidine blue (TB) or used for immunohistochemistry (IHC).

A range of morphological parameters were assessed on the HE and TB stained sections and scored as follows: completeness of excision (0 = complete; 1 = incomplete), local growth pattern (1 = nodular; 2 = multinodular; 3 = multinodular to infiltrative; 4 = infiltrative), vascular invasion (0 = absent; 1 = present), as well as neoplastic mast cell granularity (1 = mild; 2 = mild to moderate; 3 = moderate; 4 = moderate to marked; 5 = marked), anisokaryosis (1 = mild; 2 = moderate; 3 = marked), nuclear pleomorphism (0 = absent; 1 = present), and presence of binucleated neoplastic cells (0 = absent; 1 = present). The mitotic index (MI) was established as the number of mitotic figures per 10 randomly chosen representative high power fields (HPF; 400x).

Immunohistochemistry (IHC)

IHC served to assess the KIT and PCNA expression in neoplastic mast cells. Briefly, after deparaffinisation and rehydration, sections were incubated in EDTA pH 9.0 in a microwave oven (4 min at 350 watts, followed by 15 min at 650 watts) for antigen retrieval. Endogenous peroxidases were blocked with Dako Real Peroxidase-Blocking Solution (Dako, Glostrup, Denmark). Sections were then incubated for 15-18 h at room temperature with rabbit anti human-KIT (Dako, Gostrup, Denmark; 1:500 in Tris buffered saline and Tween 0.05% (TBST)) or mouse anti-PCNA (clone PC10, Dako; 1:100 in TBST), known to cross-react with equine KIT (Clarke *et al.,* 2014) and PCNA (Maja *et al.,* 2013) respectively. After incubation with an anti-rabbit or anti-mouse polymer based detection system (Envision Plus, Dako), the peroxidase reaction was developed with diaminobenzidine (Impact DAB, Vector Labs Inc., Burlingame, USA), followed by counterstaining with Papanicolaou's haematoxylin. Consecutive sections incubated with non-immune rabbit serum or a murine subclass matched unrelated primary monoclonal antibody served as negative controls. Sections of a normal equine dermis with a few non-neoplastic mast cells and sections from an unaltered jejunum (containing Cajal's cells) served as positive controls for KIT, while sections of lymph node and epidermis were used as positive PCNA controls.

The KIT expression pattern was determined according to parameters published for canine CMCT (Webster *et al.,* 2004). To specify: With KIT staining pattern I, the majority of neoplastic cells exhibited a membrane reaction, occasionally together with faint cytoplasmic staining; staining pattern II was represented by an intense, focally clustered cytoplasmic KIT reaction or a strong stippling throughout the cytoplasm, and staining pattern III by diffuse cytoplasmic granular staining in mast cells that obscured all other cytoplasmic features.

Tumours in which more than one pattern was seen (mixed pattern) were classified according to the predominant pattern.

PCNA expression was seen predominantly in the nucleus (non-mitotic proliferating cells) or, more rarely, the cytoplasm (cells undergoing mitosis), and the average number of PCNA-positive cells per 10 randomly chosen representative HPF was used as the PCNA index.

Follow-up study and statistical examinations

A retrospective follow up survey form was sent to the veterinary surgeons who had submitted the diagnostic specimens. The form included questions regarding the recurrence of the neoplasm after surgical excision, the disease-free survival time, and the overall survival of the patient. An attempt was made to correlate the follow up data with the histological data (i.e. KIT staining patterns, morphological features, proliferation indices).

The statistical association between morphological parameters, proliferation indices and KIT staining patterns was investigated with the Chi-square (for category variables) or Mann-Whitney (for numerical variables) statistical tests, using the SPSS 13 Software (SPSS 13.0, SPSS Inc, IBM Chicago, USA). Correlation between MI and PCNA score was determined using Pearson's Rho (R). Statistical significance was based on a 5% (0.05) level.

Results

Tumours mainly originated from the head (22/45; 48.8%), some from the legs (6/45; 13.4%) and trunk (3; 6.7%); in 14 cases (31.1%), information on the location was not available. The age of affected animals at the time of CMCT diagnosis ranged from 3 to 13 years (average: 12 years). The majority of affected horses were geldings (25/45; 55.6%), among the remaining patients were 3 (6.7%) stallions and 12 (26.7%) mares, in the remaining 5 cases (11%), information on the gender was not provided. Arabian (10) with Arabian cross (1) (11/45; 24.4%) and Thoroughbred (5) with Thoroughbred cross (6) (11/45; 24.4%) horses were most frequently affected. Other affected breeds included Warmblood (3), French breed (2), Irish (2), Connemara (1), Irish Sports Pony (1), Appaloosa (1), Native bred (1), Standard bred (1), and Coloured Cob (1); in 10 cases (22.2%), the breed was not known.

The histological examination of the surgically excised tissue showed that the vast majority (41/45; 91.1%) of the infiltrates were incompletely excised, in only 8.9 (4/45) did they not extend to the surgical margins. Neoplasms varied in size (2-15 cm diameter) and were characterised by a predominantly multinodular or more diffuse and locally infiltrative growth of mast cells with variable numbers of infiltrating eosinophils. There was no evidence of blood or lymphatic vessel infiltration. The cytoplasmic granularity of the mast cells varied and did not exhibit a dominant pattern, as identified on the TB-stained sections. Binucleated cells were only detected in one case, where they were seen in small numbers. In two thirds (30/45) of the cases, the mast cells exhibited small (7-10 µm in diameter) hyperchromatic nuclei with compact chromatin and inconspicuous nucleoli. They generally showed only mild anisokaryosis (score 1) and no nuclear pleomorphism (score 0). Based on these criteria, these CMCT were grouped as "well differentiated cutaneous mast cell tumours" (WD-CMCT, Fig. 1A). In contrast, in the remaining one third (15/45) of tumours, the mast cells exhibited nuclear pleomorphism (score 1) and moderate or marked anisokaryosis (scores 2 or 3). These were grouped together as "poorly differentiated cutaneous mast cell tumours" (PD-CMCT, Fig. 1B; Table 1). Furthermore, local infiltrative growth was significantly associated with PD-CMCT (Table 2).

The degree of mast cell proliferation was assessed based on the mitotic index (MI) and the amount of PCNA positive cells. MI and PCNA scores showed a significant positive correlation (R: 0.829, $P<0.01$). Within the entire study population, the median of the MI and PCNA score was 0 (range: 0-12) and 11 (range: 0-150), respectively; in 60% of the cases (27/45), mitotic figures were not observed at all. In WD-CMCT, the PCNA score median was 2 (range: 0 to 50; Fig. 1C) and the MI median was 0 (range: 0-6), whereas in PD-CMCT, it was 49.5 (range 1 to 150; Fig. 1D) and 3.5 (range: 0-12), respectively (Table 2), confirming a significant difference ($P<0.001$) in the proliferative activity of the mast cells in both tumour groups.

KIT was expressed by both normal mast cells in the positive control sections and the mast cells in all CMCT. The normal dermal mast cells, like the Cajal's cells in the jejunum, consistently exhibited a peripheral membrane reaction. This was also observed in the mast cells of the majority of CMCT (39/45; 86.7%; KIT pattern I; Fig. 1E). However, in two of the cases classed as PD-CMCT (4.4% of all cases; 13.3% of the PD-CMCT), a focally stippled perinuclear cytoplasmic reaction (pattern II) was seen, and in another 4 PD-CMCT (8.9% of all cases; 26.6% of the PD-CMCT) a diffuse granular cytoplasmic reaction (pattern III; Fig. 1F) was observed. The outcome of the assessment of the KIT staining pattern in relation to the morphological features is summarised in Table 1, and the significant correlation with poor mast cell differentiation in Table 2. KIT patterns II and III were significantly correlated with a higher anisokaryosis score, both when KIT patterns I and III were compared with each other ($p< 0.05$) (Fig. 2A) and when KIT patterns II and III were compared with KIT pattern I ($p<0.01$) (Fig. 2B). Similarly, a significant difference in MI or PCNA score was observed between KIT pattern I and III (Fig. 2C, E) or KIT pattern I and II+III cases (Fig. 2D, F).

Complete follow-up information was obtained from the referring clinicians in 9/45 cases (20%). Among these were 6 tumours with KIT pattern I (4 WD- and 2 PD-CMCT), of which 3 (2 PD- and 1 WD-CMCT) had recurred, in one PD-CMCT case within 1 month and the second within 2 years; for the WD-CMCT, information on the time scale was not provided. The one PD-CMCT with KIT pattern II (PCNA score: 25, MI: 1) from which follow-up information was obtained had recurred at the site of surgery within 1 month. Also, one of the two PD-CMCT with KIT pattern III (PCNA score: 83, MI: 4) with complete follow-up information had recurred and the horse died 26 month after surgery for unknown reasons. Eight animals (88.8%) were alive when the study was completed.

Discussion

The present study investigated the morphological characteristics, proliferative activity and immunohistochemical KIT expression pattern in equine CMCT. It was performed in an attempt to gather data on a potential heterogenicity of equine CMCT with regards to differentiation and clinical behaviour and based on the assessment of features known to reflect the behaviour of CMCT in other species (Webster et al, 2004; Kiupel *et al.,* 2011). .

The average age of affected horses was 12 years, similar to that recorded in a recent study (Clarke et al, 2014); however, the generally wide age spread confirms that CMCT can develop at any age in horses. Like others before, we also found male horses to be more frequently affected than mares (Scott & Miller, 2011; Clarke *et al.,* 2014). Breed predilections have only recently been suspected, in a study where Arabians were identified as being 5.1 times more at risk of developing a CMCT than other equine breeds (Scott & Miller, 2011; Clarke *et al.,* 2014). We observed CMCT most frequently in Arabian (cross) and Thoroughbred (cross) horses, but without statistical risk association. The head has been reported as the predominant site for equine CMCT (Valentine, 2006), and this was also the site most frequently affected in our case series, followed by the legs and trunk.

Histologically, our CMCT population was in the majority (two thirds) represented by infiltrates of well differentiated mast cells, predominantly without any morphological evidence of malignancy. We subsequently addressed these tumours as WD-CMCT. However, one third of the cases was composed of less differentiated mast cells and exhibited some morphological features of malignancy, including a tendency for local infiltrative growth; we classified these as PD-CMCT. The latter differed significantly from the WD-CMCT also in terms of their proliferative activity. Based on the criteria that we assessed, WD- and PD-CMCT could easily be discerned. Interestingly, a recent study on a larger equine CMCT cohort conducted in the USA did not find evidence of such a diversity, but reported all 72 tumours in their study to be composed of well differentiated mast cells, with only "minimal" anisocytosis, anisokaryosis and mitotic figures (Clarke *et al.,* 2014). Several factors may be responsible for the differences between the results of the two studies. Firstly, Clarke and co-authors applied different inclusion criteria; while a large proportion of lesions (44%) in their study contained only an "estimated percentage of mast cells in the lesion" of up to 25%, we have excluded processes in which mast cells represented a minority (<50% of the section area) to avoid inclusion of potentially non-neoplastic mast cell rich processes (Scott & Miller, 2011). Unfortunately, the lack of photographic documentation of the previous publication does not allow a direct comparison (Clarke *et al.,* 2014). Secondly, it is possible that different morphological grading systems were applied. However, since specific information on scores or measurements used for the qualitative and/or (semi)quantitative assessments is not provided in the published paper (Clarke *et al.,* 2014), a direct comparison is, again, not possible. In any case, this recent study reported only 3 cases (4%) with more than 2 mitotic figures per 10 HPF (Clarke *et al.,* 2014), a cut off MI that would in our study identify 13

CMCT (29%), the majority of which (10/13) belong to the tumours classified as PD-CMCT. Thirdly, it cannot be excluded that epidemiological and genetical differences between the two study populations from the USA and Great Britain, respectively, could account for the discrepancy between the results of the two studies.

Our immunohistochemical assessment of the KIT expression patterns in neoplastic cells yielded results that were very similar to those obtained in the previous study, although we observed a slightly higher frequency of patterns II and III (15% vs. 12%) (Clarke *et al.,* 2014). However, when we evaluated KIT expression and proliferative activity together, we observed that CMCT with KIT expression patterns II and III all had a high proliferative activity.

In the aforementioned study the authors conclude that the KIT expression pattern is not associated with clinical outcome or tumour behaviour and gain their conclusions from data generated by a retrospective follow-up study that was designed in a similar way to ours. The authors had obtained relevant follow-up information in 30% of their cases (n=20/72) (Clarke *et al.,* 2014). Unfortunately, we were only able to gather complete follow-up information on 20% of our cases. It is our opinion that the numbers of both studies are too low to be fully representative of the whole study population, since the clinical behaviour of the majority of cases (70% and 80% respectively) is completely unknown. However, it is interesting to note that 4 of the 5 CMCT that we found to have recurred were PD-CMCT, and among these was the only horse that died before the end of the retrospective follow up study. In our opinion, larger and prospective studies are needed to further comment on the behaviour and potential associated morphology, proliferative indices and/or KIT expression pattern as potential prognostic markers for equine CMCT. However, based on our observations, KIT translocation from the cell membrane to the cytoplasm is a phenomenon that is only seen in a subgroup of equine CMCT with a lower degree of mast cell differentiation, morphological features of malignancy and a higher proliferative activity.

In canine CMCT, *c-kit* mutation has been reported (Yamada *et al.,* 2011), but evidence of an association between *c-kit* mutation and an abnormal c-KIT protein expression pattern has been inconsistent (Fett *et al.,* 2013; Webster *et al.,* 2004). Our immunohistochemical results suggest that such mutations may also occur in equine CMCT, and more frequently than previously anticipated, as there is some evidence of an association between various mutations in this gene and a white colour coat phenotype in several horse breeds (Haase *et al.,* 2009). Whether these mutations are also associated with abnormal immunohistochemical KIT expression in horses, or specifically in CMCT, remains to be determined, and further studies are needed to investigate this in more detail.

Conclusions

Our study identified a subpopulation of poorly differentiated equine CMCTs with morphological features of malignancy, a higher proliferative activity and abnormal KIT expression. Although the clinical follow-up data was too limited to allow a valid assessment of the relationship between morphological features, proliferation, KIT expression patterns and prognosis in equine CMCTs, the results provide a rational basis for further research in this area, confirm that at least a certain proportion of processes that are currently classified as equine CMCT are true neoplastic processes, and support the hypothesis that a proportion of CMCTs is at least locally aggressive.

Conflict of interest statement

None of the authors has any financial or personal relationships with third parties that could inappropriately influence or bias the content of the paper.

Ethics statement

This project has been approved by the University of Liverpool, Veterinary Research Ethics Committee (Ref: VREC49).

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Figure Legends

Fig. 1. Morphology , KIT and PCNA expression in equine well differentiated (WD-) and poorly differentiated (PD-) cutaneous mast cell tumours (CMCT).

A. WD-CMCT). Densely packed neoplastic cells with scattered eosinophils and mild anisokaryosis. **B**. PD-CMCT. The neoplasm is composed of densely packed round cells that exhibit moderate to marked anisokaryosis (arrow) and mitotic figures (arrowhead). HE stain. Inset: The presence of abundant metachromatic granules within the cytoplasm of the neoplastic cells confirms these as mast cells. Tolouidine blue stain. **C**. WD-CMCT. PCNA-positive proliferating cells (arrowheads) are rare. **D**. PD-CMCT. A large number of PCNA positive, proliferating cells is seen. **E**. WD-CMCT with KIT pattern I. Mast cells exhibit membranous KIT expression. **F**. PD-CMCT with KIT pattern III. Mast cells exhibit granular cytoplasmic KIT expression. C-F: Indirect immunoperoxidase, Papanicolaou's heamatoxylin counterstain. Scale bars = 20 µm.

Fig. 2. Association of KIT expression and the presence of anisokaryosis, the mitotic index and the PCNA score in equine CMCT.

Box and whisker plots to illustrate the association between median anisokaryosis score (A, B), mitotic index (C, D) and PCNA score (E, F) with KIT patterns I, II and III (A, C, E) or KIT patterns I and II + III (B, D, F). Circles represent outlayers. * = $P < 0.05$. ** = $P < 0.01$.